The principal mechanism underlying megalencephalic leukoencephalopathy with subcortical cysts, implications for white matter water homeostatis

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Introduction and aims of this thesis

1. Introduction

Before the introduction of magnetic resonance imaging (MRI) as a diagnostic tool into medicine, hereditary childhood white matter disorders were most often diagnosed at autopsy. With the introduction of MRI, white matter disorders could be visualized in detail in living patients for the first time [1-3].

In the following years it was found, however, that the majority of children with white matter disorders remained without a specific diagnosis [4]. As a consequence families with children suffering from these disorders often visited doctor after doctor hoping for a diagnosis but remained in the dark. Without a diagnosis, no information about the disease outcome, specific treatment or prenatal diagnosis could be offered to these families.

In the beginning of the nineties it became clear that different disorders presented with different patterns of MRI abnormalities and MRI pattern recognition was developed [3-5]. Using MRI pattern recognition, it proved possible to define novel white matter disorders based on their specific pattern of MRI abnormalities [4]. Genetic analysis in defined groups of patients led to the discovery of genes associated with several of those disorders [6-10]. Today children with these disorders leave the hospital with a diagnosis and parents are offered prenatal diagnoses for future family planning.

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is one of these hereditary childhood white matter disorders that was characterized by MRI [11] and for which a responsible gene, *MLC1*, was identified [7]. MLC patients develop macrocephaly within the first year of life. After several years, slowly progressive cerebellar ataxia, spasticity and epileptic seizures occur. Most children become wheelchair-dependent as teenagers. The mental capacities of patients remain relatively intact. MRI reveals diffuse signal abnormalities and swelling of the cerebral white matter and subcortical cysts [11] (Figure 1A and B). Quantitative MRI parameters indicate that the white matter water content is highly increased [12]. Brain biopsies reveal that the increased water content is due to increased extracellular spaces and vacuoles in the outer lamellae of the myelin sheaths [13-15] (Figure 1C and D). About 25% of MLC patients do not have mutations in *MLC1* and further genetic analysis indicates that there is at least one other gene involved in the disease [16-18]. Although the genetic defect in most MLC patients has been found, the function of the MLC1 protein and the mechanisms underlying the leukoencephalopathy remain unknown. Determining the function of the MLC1 protein and finding other genes involved in MLC may provide valuable information for future development of specialized MLC treatment.

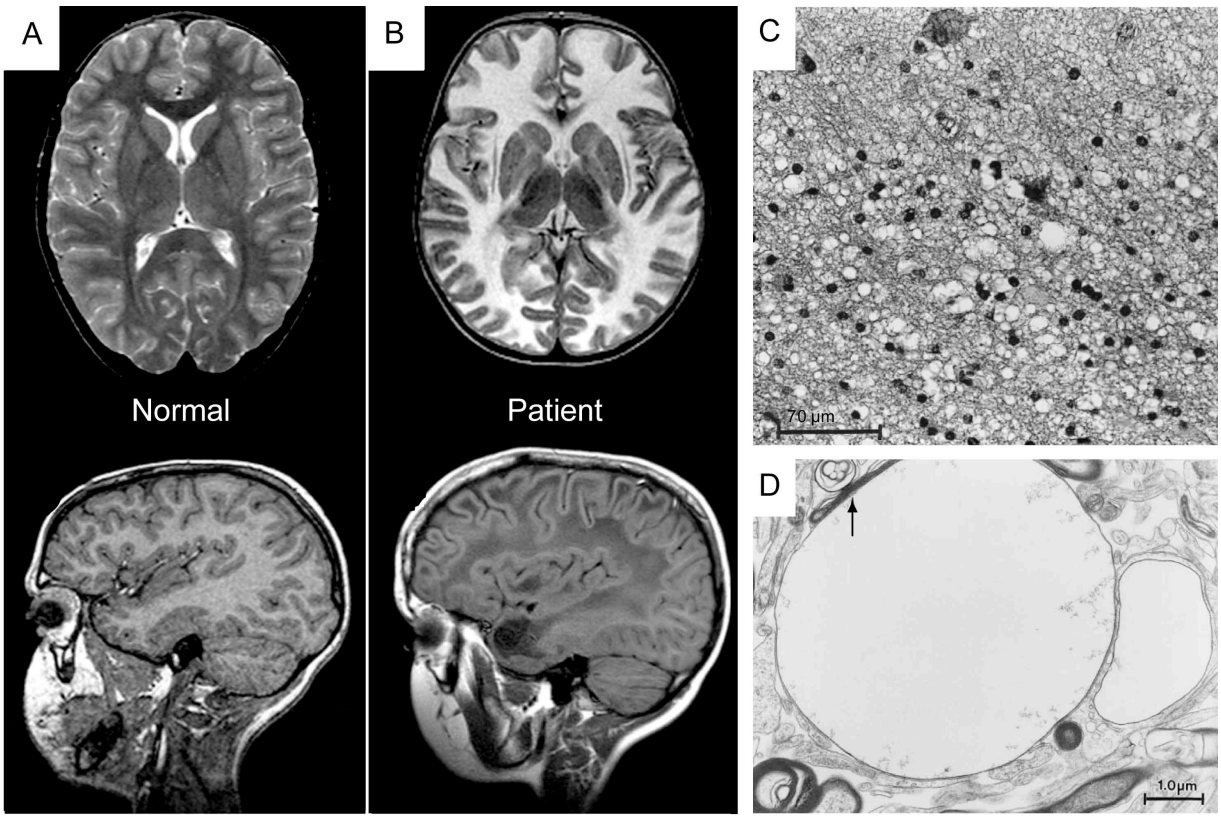

Figure 1: **Diffuse white matter abnormalities and myelin vacuolation in MLC.** Brain MRIs of a control person (**A**) versus a MLC patient (**B**). The MRI of the MLC patient displays diffusely abnormal, slightly swollen cerebral white matter and subcortical cysts in the anterior-temporal subcortical region (observable in bottom MRI). Light microscopic image of a brain biopsy of a MLC patient (**C**). The left upper part displays unaffected neocortex and the bottom part shows densely vacuolated white matter. Electron microscopic image of brain biopsy of a MLC patient (**D**) showing two membrane bound vacuoles. The large vacuole is partially covered by a myelin sheath (arrowhead). Figure adapted from (Van der Knaap, 1996)

1.1. MLC1 protein

The *MLC1* gene was identified through classical positional cloning and mutation analysis in 2001 [6,19] . It encodes MLC1, a 377 amino acid plasma membrane protein with eight transmembrane domains; both termini have an intracellular location [20] (Figure 2). The protein is solely expressed in vertebrates, the myelin producing organisms, and is highly conserved throughout species [20]. Humans leukocytes express low levels of MLC1 [20], but MLC1 is almost exclusively expressed in the brain [21-23].

MLC1 is a so-called orphan protein that does not belong to a known family or class of proteins. Up to the start of this thesis its function has remained unknown. MLC1 oligomerizes in *vivo* [24]. Amino acid sequence homology analysis reveals weak similarity with potassium channel Kv1.1, ABC-2 type transporter and sodium:galactoside symporters [7,21] and MLC1 contains an internal repeat that is also found in several ion channels [24]. The mainstream hypothesis is, that MLC1 functions as anion channel or a transporter [7,21,24].

1.2. MLC1 expression pattern

Northern blot analysis has shown that MLC1 mRNA is found in various brain regions [21]. Immunohistochemical and in situ hybridization studies, performed in humans and mice, show that MLC1 is predominantly expressed by glia, including astrocytes, Bergmann glia and ependymal cells, but not by oligodendrocytes and microglia [20,24,25]. MLC1 has also been found in some axonal tracts [24,25]. A fluorescent immunohistochemical study has shown that in astrocytes MLC1 is predominantly located in so-called astrocytic end-feet contacting perivascular, subependymal and subpial regions [20]. Higher magnifying electron microscopy (EM) studies show that MLC1 is located in astrocyte-astrocyte contacts [24].

1.3. MLC patient mutations

Approximately 50 different mutations have been identified in the years following the discovery of the MLC1 gene [7,16,17,26-28]. These mutations are distributed evenly through-out the entire gene (Figure 2). About half of these mutations are missense mutations and may result in reduced protein expression at the plasma membrane as shown in an *in vitro* assay [24]. The other half mainly consists of splice-site mutations or insertions and deletions. Only one nonsense mutation has been reported [7].

With a few exceptions, almost all mutations are private mutations. There is an evident common founder effect mutation in the Indian Agarwal community. The patients in this community share the same frameshift mutation leading to a premature stop early in the protein [29,30].

MLC patients show variability in severity of clinical symptoms and disease progression [7,30]. So far no genotype-phenotype correlation has been found [18]. Even within individual families, there is a variety in clinical course and severity. Environmental factors and genetic modifiers could be the cause of this variety in disease phenotype.

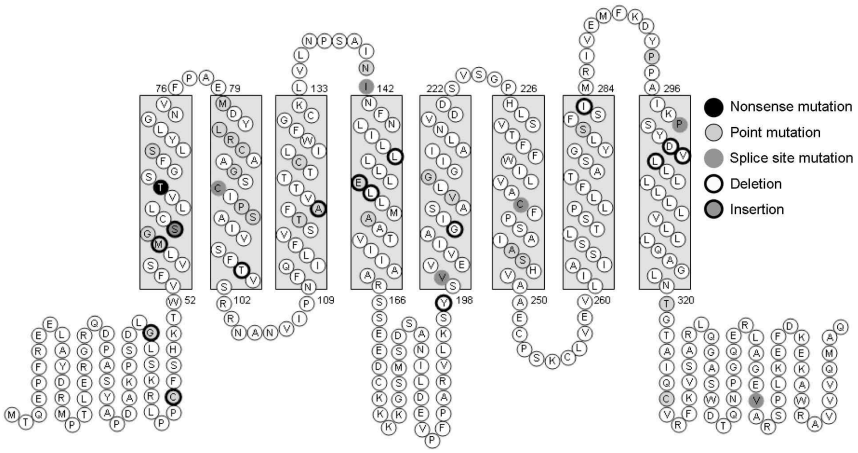

Figure 2: **MLC1 protein amino acid sequence drawing** showing the eight transmembrane domains and the intracellularly located N- and C-termini. Different mutations are found spread throughout the protein (Boor, 2006).

2. White matter: myelin, glia and development

Areas in the brain and spinal cord containing large amounts of myelinated axons are called white matter. Its name is derived from the high lipid content of myelin that makes white matter look white. As MLC is primarily a white matter disorder, this second paragraph will focus on myelin, the cells of the white matter, and the development of the white matter.

2.1. Myelin

Myelin makes up most of the substance of the white matter in the central nervous system. It is a spiral membranous structure formed by oligodendrocytes. Long extensions from oligodendrocytes are tightly wrapped around axons, the cytoplasm is squeezed out, the touching inner sides of the membranes fuse to form the major dense lines (see below) and the touching outer sides of the membranes become closely apposed and form the intraperiod line (see below). Generally, the larger the diameter of the axon the thicker the myelin sheath.

Like other biological membranes, myelin's primary structure is a lipid bilayer, but unlike other biological membranes this lipid bilayer contains large amounts of long chain saturated fatty acids. The presence of these saturated fatty acids enables the high density of the myelin and ensures the tight packing of axons. Besides the abundant lipids, the myelin membrane also contains proteins. Some proteins are believed to be important in communication between the axon and the inner lamellae of the myelin sheath or between glia and the outer lamellae; others fuse and stabilize the layers. Proteins represent about 20% of the dry mass of myelin. The proteins on both sides of the cell membrane are visible as dark lines by electron microscopy. Electron microscopic examination of myelin shows darker lines alternated by two less dark lines (which are sometimes seen as only one line). The darker lines are the so-called major dense lines and represent the fused inner protein coats of the cell membrane, whereas the two less dark lines are the so-called intraperiod lines and represent the closely apposed outer protein coats of the cell membrane.

Myelin is crucial to propagate electrical signals efficiently [31]. The myelin sheath does not run along the entire length of axons but shows discontinuations known as nodes of Ranvier. The nodes of Ranvier contain ion channels and transporters involved in action potentials, like voltage-gated sodium channels. Through the presence of myelin segments, action potentials jump from one node of Ranvier to the next, referred to as salutatory conduction. In general, the internodal segments become larger with increasing axon length. The myelin bordering these internodal segments is spirally rolled up and contains cytoplasm (paranodal loops). Areas with cytoplasm are also found in the compact myelin sheath more distal from the internodal segments and are referred to as Schmidt-Lanterman incisures. These structures

with remaining cytoplasm contain proteins involved the transport of water and small molecules [32].

2.2. Cells of the white matter

Besides the large quantities of myelinated axons, the white matter contains several types of glia. These cells can be divided into two major groups, macroglia and microglia. In the central nervous system (CNS), macroglia can be further divided into astrocytes, oligodendrocytes, ependymal cells and radial glia. These macroglia develop from the ventricular zone of the neural tube.

2.2.1. Microglia

Microglia are described here only briefly, as they fall outside the scope of this thesis. They originate from outside the nervous system and are physiologically and embryologically unrelated to the other cells in the nervous system [31]. Microglia originate from the bone marrow and are the resident macrophages of the brain and spinal cord. They are the first and main form of active immune defense in the CNS. Microglia are small and when activated have processes that are stouter and more branched then those of inactive cells.

2.2.2. Astrocytes

Astrocytes account for one third of the brain mass. They are the predominant cell type within the CNS and perform many functions. Metabolically supporting neurons [33,34], regulating ion concentrations in the extracellular space [35,36], volume regulation, promoting myelination activity by oligodendrocytes [37] and filling in the gaps after brain injury with “glial scar” [38] are only a few examples. Astrocytes are large cells with irregularly shaped cell bodies and leaflet cellular processes. Astrocytes are generally divided into two types: fibrous and protoplasmic [39]. Fibrous astrocytes are usually located within the white matter, have relatively few organelles, and exhibit long, unbranched cellular processes. Protoplasmic astrocytes are found in the gray matter and possess large quantity of organelles and exhibit short and highly branched cellular processes.

Astrocytes are usually coupled through gap junctions that interconnect astrocytic processes to form large intercellular networks. This coupling enables astrocytes to communicate for long distances and redistribute molecules to prevent local accumulation. This process is known as “spatial buffering”. Astrocytic processes are not merely interconnected with each other but have so-called “endfeet” that associate with other cell types, synapses, blood vessels (perivascular endfeet), and cerebrospinal fluid (CSF) barriers (subpial and subependymal endfeet). Astrocytes with endfeet that connect to synapses modulate synaptic

activity through the secretion [40,41] or uptake [42,43] of molecules that influence synaptic transmission. Astrocytes with perivascular endfeet play a role in the development and maintenance of the blood-brain barrier [44,45] and contribute to the regulation of the cerebral blood flow [46,47]. Astrocytic endfeet directed to the brain-blood or brain-CSF barriers are equipped with specialized water and ion channels that enable astrocytes to regulate water and ion concentrations in the brain (Chapter 4).

2.2.3. Radial glia

Radial glia are often classified as astrocytes because they show similarities in protein expression like glial fibrillary acidic protein (GFAP), which is regarded as a marker for astrocytes. Radial glia play a pivotal role during brain development. They guide migrating neurons and direct neuronal outgrowth but also act as precursor cells for neurons and astrocytes. They have long radial processes extending from the ventricular zone to the pial surface. In the adult cerebellum they are known as “Bergman glia”. In the retina they are the principal glia and known as “Müller cells”. [48]

2.2.4. Ependymal cells

Ependymal cells form the protective cell layer that lines the brain ventricles and the central canal of spinal cord. Ependymal cells are simple cube-shaped epithelium cells that may contain cilia on their apical surfaces. These cilia help to circulate CSF around the CNS, move cellular debris in the direction of bulk CSF flow, and optimize the dispersion of neural messengers in the CSF [49]. Their apical surfaces are also covered with microvilli, which absorb CSF. Mice with loss of their ependymal cilia develop hydrocephalus [50]. Tight junctions between ependymal cells control fluid release across the cell layer. Modified ependymal cells, called “choroid plexus cells”, produce and secrete CSF at the choroid plexus. Ependymal cells are related to astrocytes, they arise from the same astrocyte progenitor cells and also express GFAP [51].

2.2.5. Oligodendrocytes

Oligodendrocytes are the key cells in myelination. Oligodendrocytes form flat cell processes, which wrap around the nerve axon in a spiral fashion [52]. Satellite oligodendrocytes form an exception; they are located perineuronally and may serve to regulate the microenvironment around neurons [53]. Oligodendrocytes are smaller in size and have a greater density of both the cytoplasm and nucleus than astrocytes. One oligodendrocyte provides myelin for multiple axons (up to 50) and one axon contains myelin from numerous oligodendrocytes. The ratio between cell body surface membrane and myelin membrane is estimated at 1:620. The

production and maintenance of this vast amount of myelin membrane requires the optimal coordination of lipid and protein interaction and synthesis.

2.3. White matter development

The brain of a newborn weighs around 400 grams. At one year of age the brain weighs almost 1,000 grams. By 2 years of age the brain has reached 80 percent of its adult size and weighs about 1100 grams. Myelination of the brain is one of the major processes that account for this growth. Myelination begins early in the 3rd trimester of pregnancy with the most rapid period of myelination occurring in the first two years of life. The myelination process follows a specific time course and pattern [54]

With myelination the lipid composition of the white matter changes a lot in the first two years of live. The relative amount of glycolipids increases whereas the relative amount of phospolipids shows a decrease. Although the lipid composition in the white matter as a whole changes, the composition of the myelin present at birth has the same lipid composition as that of a two year old [55]. The major difference between white matter in young infants and adults is the quantity of myelin rather than its quality. Another important difference in the white matter between a young infant and adults is the water content. The water content in the brain decreases over time and shows a larger decrease in the white matter compared to the gray matter. The water content of neonatal gray matter is about 89% and of neonatal unmyelinated white matter about 87%, whereas the water content of adult gray matter is estimated to be 82% and of adult myelinated white matter 72%.

3. Water and ion regulation in the brain

All organisms have a diversity of mechanisms to maintain water homeostasis under numerous physiological conditions [56]. Because water flows from compartments with low osmolarity to compartments with a higher osmolarity, water homeostasis stringently coincides with regulation of osmolarity or ion homeostasis. Water flows against a concentration gradient, consequently most mechanisms involved in water homeostasis involve the transport of osmolytes. The increased brain water content and numerous fluid-filled vacuoles in MLC patients suggest disruption of brain water homeostasis. This chapter will describe general challenges for water homeostasis in the brain, sources and distribution of water and ions in the brain and the mechanisms in which water crosses plasma membranes. Finally, this chapter wills discuss the general mechanisms involved in cell volume regulation.

3.1. Water homeostasis in the brain

Maintaining water homeostasis is essential for all organisms. Although water homeostasis is important in the entire human body, it is of utmost importance in the brain. The rigid skull that protects the brain from the outside world becomes the brain's worst enemy when confronted with disruption of brain water homeostasis. Increased brain water content leads to increased intracranial pressure, which compromises brain function and can cause death. This is in clear contrast with other organs, which may similarly increase in volume without serious consequences.

The high metabolic and high electrical activity of the brain forms a major challenge for maintaining brain water homeostasis. The brain consumes about 25% of the body's glucose, which consequently changes the brains osmolytes and water content. The high electrical activity of the brain induces vast shifts in intra- and extracellular ion concentrations and thus water [31,57]. In order to sustain functional neuronal firing and thus brain function, rapid and accurate re-uptake and extrusion of these ions and water is pivotal [58]. Not surprisingly, water and ion homeostasis in the brain is a tightly regulated process. Additionally, during the development of the brain vast amounts of proteins and lipids need to be produced and transported to myelinate billions of axons[54]. All these processes require intensive uptake from and secretion to the vascular system, while maintaining water and ion homeostasis.

3.2. Sources and distribution of water and osmolytes

Brain water is distributed over blood, CSF, interstitial and intracellular compartments. The electrolytes Ca^{2+}, Na^{+}, K^{+} and Cl^{-}, amino acids, organic acids and sugars are the main osmolytes and their distribution between compartments is tightly regulated. Like other cells in the body, brain cells are bathed in interstitial fluid (ISF). Changes in the osmolarity of ISF consequently cause cells to swell or shrink. Unlike the rest of the body, brain ISF does not simply diffuse to and from the vascular system due to the blood-brain barrier (BBB). Most of the brain ISF comes from the vascular system and from the CSF. Evolutionary studies show that higher evolved species have a better BBB and as a consequence a more stable ISF composition. [59].

The means by which water flows across the blood-brain and CSF-brain barriers are highly regulated and involve many known and unknown cell-cell interaction proteins, channels and transporters. Besides taking up water from its surroundings, the brain itself also produces water. When glucose, the main energy substrate of the brain, is metabolized, a substantial amount of water is produced [60]. In contrast to water derived from blood or CSF, this water is not retained to specific brain compartments by a specialized barrier. Most prominent perhaps is the vast amount of water and ions that are constantly redistribution as a consequence of

neuronal action potential firing [31,57]. As water flows against its concentration gradient, water will be redistributed alongside neurotransmitter and ions during neurotransmission. These ions, neurotransmitters and water need to continuously be retaken up from the extracellular space in order for the brain to sustain its electrical activity [31,58].

3.3. Water across plasma membranes

Cells are highly permeable to water. In general there are three distinct ways in which water can pass through plasma membranes [61]. The first way is by direct diffusion of water through the lipid bilayer. It was long assumed that the transport of water through the lipid bilayer occurred only in this manner. However, water also can pass through the plasma membrane in co-transport with either organic or inorganic ions. Osmolytes are constantly transferred between the intracellular and extracellular environment of the cell. Water is often co-transported with these osmolytes. For example, the glutamate transporter Eaat1, which is one of the most efficient water co-transporters, admits 436 ± 55 water molecules per glutamate anion [62]. The third way is by specialized water channels, the so called aquaporins (AQPs). AQPs permit passage of large quantities of water over plasma membranes. Therefore it is not surprising that AQPs are found in a subset of epithelia that have a 10- to 100-fold higher capacity for water permeation than epithelia lacking aquaporins. AQPs permit bidirectional water transport through the lipid bilayer, and its direction is generally determined by the osmotic gradient. AQPs assemble in membranes as homotetramers. Each monomer contains a water channel and consists of six membrane-spanning α-helical domains with intracellular C- and N-termini [63].

To date, three AQPs, AQP1, AQP4 and AQP9, have been clearly identified in the brain [64]. AQP1 is a common AQP that is expressed in many organs. In the brain AQP1 is abundantly expressed in the apical membrane of the choroid plexus [65]. It is also present in the brain endothelial cells, but at much lower levels than in other organs [66]. AQP1 knock-out mice show a 25% reduction in their CSF production as compared to wild-type mice [67]. AQP4 is the most abundant water channel in the brain and is highly and mainly expressed in astrocytes, although expression has also been found in the basolateral membrane of ependymal cells [68,69]. AQP4 is implicated in brain edema formation and resolution and will be discussed in chapter 4.2.1. In the brain AQP9 is expressed in astrocytes, catecholaminergic neurons and endothelial cells of subpial blood vessels [70]. Unlike AQP1 and AQP4, AQP9 not only passes water, but is also permeable to glycerol and even to various solutes, including carbamides, purines, pyrimidines, and urea [71]. As AQP9 passes more than water alone, many believe that it has a role in energy metabolism.

The relative contribution of passive diffusion through the plasma membrane, cotransport with ions and passage through AQPs on water movement depends on density, expression pattern and flux capacity of individual AQPs and cotransporters.

3.4. Cell volume regulation

Cell volume changes are the consequence of passive water fluxes brought about by changes in internal or external osmolarity. In the brain large-scale cell volume changes can result in swelling and herniation of the brain and death. Fortunately, most if not all cells are equipped with mechanisms to counteract such volume changes, bringing the cell's volume back to normal. The active process leading to cell volume recovery from swelling is termed regulatory volume decrease (RVD), whereas recovery from shrinkage is known as regulatory volume increase (RVI). Not surprisingly the main players involved in RVI and RVD are transmembrane proteins facilitating the uptake and or extrusion of osmolytes to increase or decrease cell osmolarity and concomitantly cell volume. Increased Cl^- permeability provoked by cell swelling was first demonstrated in Ehrlich ascites tumor cells [72], and in lymphocytes [73], and was subsequently found in essentially all cell types investigated. RVD mainly involves ion fluxes (Cl^- and K^+) through ion channels, whereas RVI mainly involves coupled ion transport (Cl^-, K^+ and $Na^{+)}$ through carrier proteins. Besides these ions, other small inorganic or organic molecules such as the amino acids taurine, glutamate, and aspartate, are also capable of significantly contributing to cell volume regulation. The exact contribution of different osmolytes involved in cell volume regulation may differ between cell types and physiological circumstances. AQPs may be co-expressed alongside these channels and transporters in order to facilitate the transmembrane water movement. Although the general mechanisms involved in volume regulation have long been established, the molecular identities of many of the proteins crucial in volume regulation remain unknown.

4. Brain water and ion regulation by astrocytes

The role of astrocytes in water and ion homeostasis is of special interest to this thesis, because MLC is caused by mutations in an astrocytic protein and MLC patients show a dysbalance in water homeostasis with increased brain white matter water content. This chapter describes how the interconnectivity of astrocytes and their coupling partners enable them to play an important role in brain water homeostasis. The second part focuses on the specific channels that, like MLC1, are located in astrocytic processes near the brain fluid barriers and are likely to play a role in brain water and ion homeostasis. Finally, astrocytic proteins that are associated with white matter vacuolation when mutated will be discussed.

4.1. Connexins

Astrocytes are highly interconnected with each other through gap junction channels and are therefore often regarded as a syncytium rather than individual cells. Vertebrate gap junctions allow direct intercellular diffusion of ions, water and small soluble molecules with a molecular weight up to about 500 Da [74]. Each gap junction consists of two hemichannels, one in each adjacent membrane, known as connexons. Each connexon is an oligomer of six identical connexins that dock to each other via their extracytoplasmic extremities. In coupled cells of the same histological type, connexons link to the same type of connexon in the second cell, forming homotypic gap junctions. In contrast, gap junctions formed between different cell types can either be homotypic, or they can be made up of different connexons, forming heterotypic gap junctions [32]. In the CNS, cell-specific and developmentally regulated expression of eight connexins has been demonstrated, with six of these eight exclusively expressed by the three types of macroglia [75-77]. Transgenic mice studies show that mutations in two of these connexins can cause myelin vacuolation, as seen in MLC patients (see 4.3.3)

Connexins are often associated with gap junction interacting proteins that can modulate gap junction communication. Many of these proteins, such as zona occludens, cadherins, claudins and caveolin, are cell-cell adhesion proteins that like connexins are generally found in tight junctions and adherens junctions [78].

4.1.1. Astrocyte-astrocyte coupling

Astrocytes are highly interconnected with each other through gap junction channels. Astrocytes express three sets of connexins, Cx43, Cx30 and Cx26 (listed in order of decreasing abundance). Astrocytic gap junctions alone represent up to 99% of the gap junctions in most brain regions with a single astrocyte being able to express more than 30,000 gap junction channels [77]. These gap junction channels link processes of different astrocytes but the majority of these gap junction channels (about 75%) are located between different processes of the same astrocyte forming what are defined as autologous junctions [79]. In addition, abundant gap junctions are formed between astrocytic processes that establish end-feet on blood vessels, between adjacent lamellar processes of astrocytes that ensheath synaptic glomeruli and that occur at nodes of Ranvier along myelinated fibers [80]. Astrocytic gap junctions play a very important role in ion and water homeostasis in the brain (Figure 3). They enable astrocytes to disperse water and ions from areas with high concentrations to low concentration, consequently relieving individual astrocytes from osmotic stress.

4.1.2. Astrocyte coupling partners

Astrocytes also form numerous gap junctions with oligodendrocyte somata and their initial processes, and with the outer turn of their uncompacted myelinated processes along axons. Oligodendrocytes express a different set of three connexins (Cx31.3 or rodent ortholog Cx29, Cx32, and Cx47) that are contained at the oligodendrocyte side of necessarily heterotypic astrocyte-to-oligodendrocyte gap junctions (Cx30:Cx32 and Cx43:Cx47) [32,81]. The gap junctions between astrocytes and oligodendroctes enable oligodendrocytes to get rid of excess water and osmolytes through the astrocyte syncytium (Figure 3). Gap junctions do not occur (or are extremely rare) between oligodendrocytes themselves [82].

Astrocytes also form gap junctions with the third type of macroglia, the ependymal cells. These cells only express Cx43 with which they form homotypic gap junctions with adjacent ependymal cells and astrocytes [83]. The gap junctions between astrocytes and ependymal cells enable both astrocytes and their gap junction coupled oligodendrocytes to diffuse excess water and osmolytes to the brains fluid barriers. Gap junctions do not occur or are extremely rare between any glia and neurons [77,84].

4.1.3. Water and ion regulation by the panglial syncytium

The above described vast network of interconnected glia is often referred to as the “panglial syncytium”. It is essential for brain water homeostasis as it is the only network in the brain that allows long-distance spatial buffering of osmolytes and water. More specifically, the panglial syncytium plays an important role in maintaining potassium homeostasis through its ability to spatially buffer potassium, a process also known as “potassium siphoning” (Figure 3). Excess potassium, generated as a consequence of neuronal action potential firing, moves from the extracellular space between the axon and the innermost myelin sheet to the innermost cytoplasmic layer of the myelin sheath. This excess potassium moves from the inner layer to the outer layer of the myelin sheath either circumferentially or via homotypic Cx32 gap junctions located in Schmidt-Lanterman incisures and paranodal loops. The excess potassium then moves from the oligodendrocyte (outer myelin sheet or soma) to astrocytes via heterotypic Cx47/Cx43 or Cx32/Cx30 gap junctions [32].

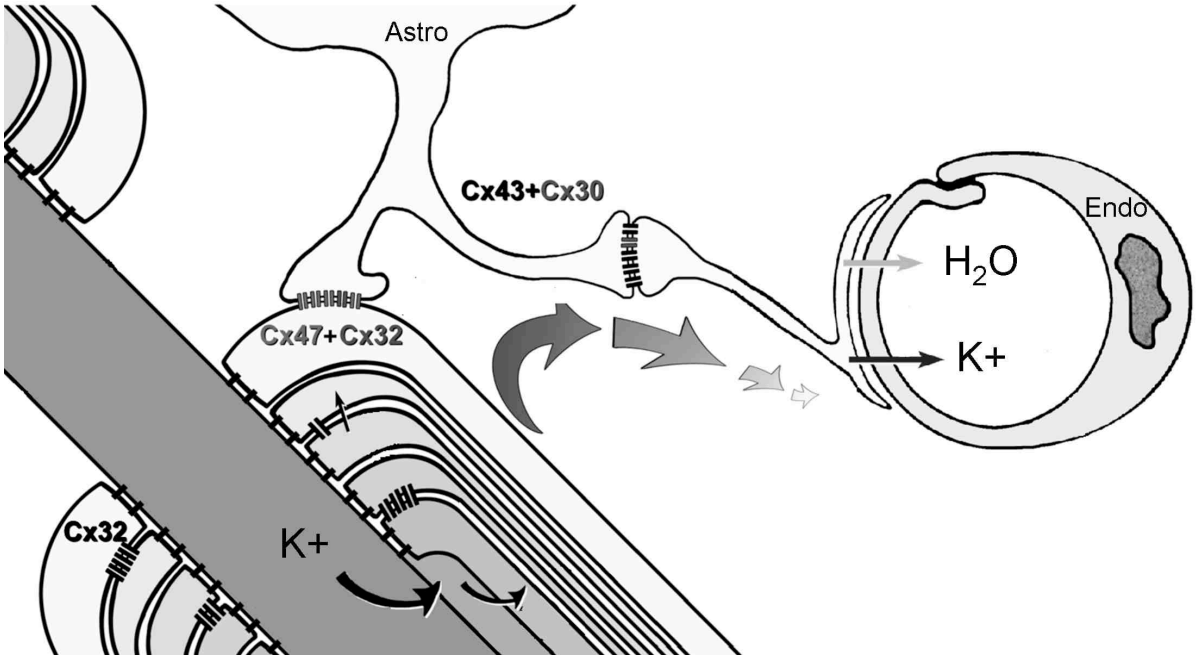

Figure 3: Connexins mediate K^+ and H_20 siphoning

Diagram depicting intra- and intercellular pathways involved in K^+ and H_20 siphoning after axonal potassium and sodium influx induced by action potential firing. Homotypic Cx32 and bi-heterotypic Cx47/Cx32: Cx43/Cx30 gap junctions pass K^+ and H_20 from the myelin into the astrocyte syncytium towards the blood-brain barrier. Both the K^+ concentration as the membrane potential decrease gradually from in the axons to the lumen of the capillary, hence the fading arrows. Figure adapted from (Kamasawa, 2006).

Once in the astrocyte, potassium diffuses along its electrical and chemical concentration gradient towards the brain fluid barriers. Thus, the panglial syncytium is specialized in maintaining brain and ion homeostasis, with a central task for the highly interconnected astrocytes. The specific channels that enable astrocytes to secrete and take up water and osmolytes from its surroundings will be discussed in the next section.

4.2. Astrocytic channels involved in water homeostasis

To fulfill their role in maintaining brain ion and water homeostasis, astrocytes are equipped with specialized ion and water channels. Like MLC1, many of these channels are present in astrocytic processes contacting CSF-brain and blood-brain barrier. This chapter will discus the various channels expressed in astrocytic processes near the brain fluid barriers and their involvement in cell volume regulation and brain water and ion homeostasis.

4.2.1. Aquaporin 4 and Kir4.1

Osmotic regulation by astrocytes involves movement of water through AQPs. AQP4 is highly and predominantly expressed in astrocytes, namely in subpial and perivascular astrocytic endfeet and to a lesser extent in endfeet surrounding synapses and endfeet in contact with

the nodes of Ranvier [68]. Water fluxes through AQP4 are bidirectional and driven solely by osmotic gradients and hydraulic pressure. Transgenic AQP4 knock-out mice show reduced brain edema after experimental stoke and hypo-osmotic stress (cytotoxic edema) [85]. However under conditions where brain edema is produced by a fluid leak (vasogenic edema), AQP4 deletion reduces the water outflow from the brain, consequently increasing edema. Astrocytic AQPs are upregulated in response to injury.

As previously mentioned, astrocytes play a pivotal role in maintaining extracellular potassium at levels compatible with continued neuronal action potential firing. The uptake and redistribution of potassium by astrocytes is primarily mediated by inwardly rectifying K^+ (Kir4.1) channels [86]. The Kir 4.1 channels are predominantly expressed in perivascular endfeet and endfeet surrounding synapses [87]. Kir4.1 also plays a role in cell volume regulation as blockage of Kir4.1 causes substantial swelling of astrocytic endfeet [88]. AQP4 has been proposed as molecular partner of Kir4.1 in spatial buffering of potassium by facilitating the consequential water movement [89]. AQP4 facilitates water uptake at endfeet surrounding synapses and the nodes of Ranvier and extrusion at the perivasculair endfeet. Superimposed on this activity-dependent water flux is a constitutive flux of water derived from the metabolic breakdown of brain glucose.

4.2.2. Volume regulated ion channels

Clearance of extracellular potassium after neuronal firing is one of many processes that can cause astrocytes to swell [90]. Several *in vitro* experiments have shown that the volume regulated anion channel (VRAC) plays a pivotal role in RVD after astrocytic swelling [91-93]. Many discrepancies exist between the reported biophysical and pharmacological properties of VRAC. The consistently reported astrocytic VRAC characteristics include the following: (I) VRAC is activated by hypo-osmotic shock and associated cellular swelling; (II) VRAC currents are mainly carried by chloride; (III) VRAC currents are outwardly rectifying; (IV) VRAC is sensitive to the chloride channel blockers, 5-nitro-2- (3-phenylpropylamino) benzoic acid (NPPB), 4-4′-diisothiocyantostilbene-2-2′-disulfonic acid (DIDS) and tamoxifen. Although VRAC currents have been extensively studied (reviewed in [94,95] all attempts to identify the molecular identity of VRAC have been unsuccessful [93].

The discrepancies concerning the biophysical properties of VRAC suggest that not one but multiple channels are responsible for the observed volume regulated anion currents. If so, VRACs may be differentially activated and expressed in different regions in the brain [96]. As the molecular identity is unknown, no evidence is available that astrocytic VRACs, like AQP4 and Kir4.1, are expressed in astrocytic endfeet near the brain-fluid barriers. Although indirect, the

fact that VRAC activity was reduced by knockdown of AQP4 [97] could point to possible co-localization in perivascular and subpial astrocytic endfeet.

4.3. Astrocytic proteins and white matter vacuolation

MLC patients display vacuoles throughout their cerebral white matter. Many mysteries remain on how astrocytes maintain brain and ion homeostasis. This section will discuss proteins that lead to the development of vacuoles in the white matter when mutated in humans or absent in mice.

4.3.1. Dystophin-associated glycoprotein complex

The dystrophin-associated glycoprotein complex (DGC) is a multi-subunit complex that forms a critical link between the cell's cytoskeleton and the extracellular matrix and is highly expressed in skeletal muscle [98]. In astrocytes, the DGC is additionally responsible for the polarized expression of proteins in endfeet. AQP4 and Kir4.1 are both anchored in the DGC through α-syntrophin [99,100]. Mutations in the *LAMA2* gene, which encodes the DGC member merosin, are associated with merosin-deficient congenital muscular dystrophy (MDC1A). Unlike MLC1 patients, MDC1A patients suffer from serious muscle weakness. Nonetheless, MDC1A patients show striking similarities in brain MRI features and pathology with MLC patients. Their cerebral white matter is also diffuse abnormal, mildly swollen and may contain anterior temporal cysts. [11,101]. Histology shows myelin vacuolation. Most likely the astrocytic DGC is involved in water homeostasis through the anchoring of ion and water channels near the blood-brain and CSF-brain barriers.

4.3.2. Chloride channel 2

Chloride channel 2 (ClC-2) is a chloride channel belonging to the ClC family. Clc-2-mediated chloride currents are activated by negative membrane voltage, cell swelling, a rise in intracellular chloride concentration or mild extracellular acidification. Although Clc-2 has been ascribed a role in volume regulation [102,103] it does not classify as a VRAC, as its current is inwardly rectifying. In astrocytes Clc-2 is expressed in perivascular endfeet and astrocytic endfeet near GABAergic axon terminals. It is suggested that astrocytes are able to siphon and deliver chloride to support inhibitory neurotransmission [104]. No human disease has so far been associated with *CLCN2;* the relationship with idiopathic generalized epilepsy could not be confirmed [105,106]. The *CLCN2*-knockout mice, however, displays widespread cerebral white matter edema and intramyelinic vacuole formation. These results suggest that a dysfunction in a chloride channel may cause a dysfunction in brain ion and water homeostasis resulting in white matter vacuolation as seen in MLC patients [107].

4.3.3. Connexins

As previously mentioned in section 4.2, connexins (Cx) are the building blocks of gap junctions and essential for intercellular communications. In the brain Cx32 and Cx47 are expressed in oligodendrocytes and mediate gap junctional communication between oligodendrocytes and astrocytes through heterotypic gap junctions (Cx32:Cx30 and Cx47:Cx43). Mutations in *GJC2* gene, which encodes Cx47, are associated with Pelizaeus-Merzbacher-like disease (PMLD), a severe hypomyelinating disorder of the CNS. [9,108]. Mutations in *GJB1* gene, which encodes Cx32 are associated with X-linked Charcot-Marie-Tooth disease (CMTX) [109]. Although a subset of Cx32 mutations cause clinical CNS manifestations [110], CMT mostly effects PNS. PMDL patients do not show evidence of brain myelin vacuolation, whereas CMTX patients may show transient, stress-provoked brain myelin vacuolation, associated with a transient encephalopathy [111,112]. Strikingly, studies performed in mice reveal that Cx47 depletion causes brain myelin vacuolation. Although the Cx32 deficient mice show no apparent phenotype, double-deficient, Cx32/Cx47 mice exhibit abundant abnormal white matter vacuolation, much more so then the Cx47 deficient mice [113,114]. These results show that dysfunction in oligodendrocyte-astrocyte gap junctions leads to abnormal white matter water content and white matter vacuolation.

5. Aims and outline of this thesis

MLC patients show a disturbance in brain water homeostasis and volume regulation portrayed by a megalencephaly due to a highly increased water content of the cerebral white matter, which appears to be mainly in intramyelinic vacuoles and possibly also partially interstitially. About 75 percent of MLC patients have mutations in the *MLC1* gene, which encodes a transmembrane protein primarily expressed in astrocytes. The function of the MLC1 protein has remained unknown since the discovery of the gene almost a decade ago. The gene or genes mutated in the remaining 25 percent of MLC patients also has or have remained unknown.

Not much is known about the cellular and molecular mechanisms behind MLC. MLC patients develop their macrocephaly within the first year of life and although the macrocephaly remains present throughout life, the head growth rate peaks in the first year of life, suggesting that the MLC1 protein plays a pivotal role during the development of the brain in the first year.

The aim of this study was to elucidate the function of the MLC1 protein, find the missing gene(s) related to MLC and determine the MLC1 expression levels during development, in an attempt to gain insights into the disease mechanisms. The following research questions have been addressed in this thesis:

1. Does MLC1 play a role in ion transport and is it involved in cell volume regulation?

Thus far, the function of MLC1 has remained unknown. Amino acid sequence analysis reveals a weak similarity with potassium channel Kv1.1, ABC-2 type transporters and sodium:galactoside symporters. Besides, MLC1 contains an internal repeat that is found in several ion channel proteins. In chapter 2 we test the hypothesis that MLC1 is involved in ion transport. This hypothesis was tested using whole-cell patch clamp recordings from different cell types with either normal, increased or reduced levels of wild type MLC1 and mutated MLC1. We subsequently tested the hypothesis that MLC1 is involved in cell volume regulation. We tested this hypothesis by loading the above mentioned cells with a fluorescent dye and imaged them during the process of cell volume recovery after hypotonic shock.

2. Which gene is associated with MLC in patients without MLC1 mutations?

Throughout the years following the discovery of the *MLC1* gene, classical genetic linkage analysis has failed to identify any other genes involved in MLC. In chapter 3 we used quantitative proteomic analysis of affinity-purified MLC1 to identify MLC1 binding partners and thus candidate genes.

3. Are MLC1 levels in the mice and human brain developmentally regulated?

All available evidence suggests that MLC1 may have its most important role during the first year of life. This is a period of most intense myelination. Up to date, studies with different results have been published on the expression levels of MLC1 during development in mice. No MLC1 expression studies performed on human material have been published. In chapter 4 we performed qPCR and Western blot analysis on samples of human brain tissue to quantify MLC1 expression levels in humans during development. In addition, we performed immunohistochemistry, western blot and qPCR analysis on transgenic MLC1 mice with an eGFP reporter gene to determine the levels of MLC1 during development.

Finally, chapter 5 will summarize and discuss the main findings of the above addressed research questions.

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